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Reverse kinetics of angiopoietin-2 and endotoxins in acute pyelonephritis: Implications for anti-inflammatory treatment?

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ABSTRACT

Based on former studies showing an antagonism between angiopoietin-2 (Ang-2) and bacterial endotoxins (LPS), we investigated the role of Ang-2 as immunomodulatory treatment. At first, kinetics of circulating LPS in Gram-negative pyelonephritis developing after urinary obstruction was studied. Serum LPS, interleukin (IL)-6 and Ang-2 were measured in 25 patients with acute pyelonephritis and sepsis before and after removal of the obstruction performed either with insertion of a pigtail catheter ($n = 12$) or percutaneous drainage ($n = 13$). At a second stage, Ang-2 was given as anti-inflammatory treatment in 40 rabbits one hour after induction of acute pyelonephritis by ligation of the ureter at the level of pelvo-ureteral junction and upstream bacterial inoculation. Survival was recorded; blood mononuclear cells were isolated and stimulated for the production of tumour necrosis factor-alpha (TNFα). The decrease in circulating LPS was significantly greater among patients undergoing drainage than pigtail insertion. This was accompanied by reciprocal changes of Ang-2 and IL-6. Treatment with Ang-2 prolonged survival from *Escherichia coli* pyelonephritis despite high levels of circulating LPS. When Ang-2 was given as treatment of *Pseudomonas aeruginosa* pyelonephritis, sepsis-induced decrease of TNFα production by circulating mononuclear cells was reversed without an effect on tissue bacterial overgrowth. It is concluded that Ang-2 and LPS follow reverse kinetics in acute pyelonephritis. When given as experimental treatment, Ang-2 prolongs survival through an effect on mononuclear cells.

1. Introduction

Acute pyelonephritis is one major cause of community-acquired severe sepsis. It is mainly caused by Gram-negative bacteria and it is commonly associated with obstruction of the renal pelvis or of the ureter by stones or neoplasms. Due to its Gram-negative origin, acute pyelonephritis accompanied by endotoxemia can be considered a prototype of pathogenesis of severe sepsis [1]. The cornerstone of treatment in these cases is removal of the obstruction by the minimal invasive techniques which is either insertion of a pigtail-like catheter under general anesthesia or percutaneous drainage under ultrasound guidance [2]. However the impact of the applied minimal invasive technique on the kinetics of released LPS from the septic renal focus as a result of the removal of kidney obstruction has never been studied.

LPS is one pathogen-associated molecular pattern that is released by the nascent cell wall of Gram-negative bacteria during bacterial replication. Through binding to its high-affinity toll-like receptor (TLR)-4, LPS elicits the production of pro-inflammatory cytokines like tumor necrosis factor-alpha (TNFα) from circulating monocytes and tissue macrophages [3]. Evidence coming from studies of our group indicates that during septic shock a circulating factor antagonizes with LPS for the release of angiopoietin-2 (Ang-2). This antagonism is partly mediated through TNFα [4]. Ang-2 has the characteristics of a double-edge sword molecule; although Ang-2 pretreatment seems to protect mice from lethal sepsis by *Pseudomonas aeruginosa* [5] endothelial production of Ang-2 mediates loss of vascular integrity and progression into shock [6].

The current study is based on the hypothesis that in the case of acute pyelonephritis after urinary obstruction change of the kinetics of LPS are accompanied by reciprocal changes of Ang-2. Using both an experimental and a clinical approach, we aimed to explore (a) how removal of the pelvo-ureteral obstruction affects kinetics of circulating LPS and Ang-2 and (b) if Ang-2 may be a promising

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candidate of immunomodulation in the event of acute pyelonephritis due to pelvo-ureteral obstruction.

2. Material and methods

2.1. Clinical study

This was a prospective study conducted in patients admitted for obstructive uropathy and sepsis in the Department of Urology of the Aghia Olga General Hospital of Athens. The study protocol was approved by the Ethics Committee of the Hospital. Patients were enrolled after written informed consent.

Inclusion criteria were: (a) at least two signs of the systemic inflammatory response syndrome [7]; (b) pyuria defined as more than 10 leukocytes/high visual field and pain on palpation at the left or right costovertebral angle; and (c) obstructive uropathy necessitating intervention defined as dilatation of the left or the right urinary pelvis more than 10 mm in renal ultrasound.

Exclusion criteria were: (a) infection by the human immunodeficiency-1 virus; (b) neutropenia defined as less than 1000 neutrophils per mm^3 of blood; and (c) chronic intake of corticosteroids defined as more than 0.5 mg/kg of equivalent prednisone daily for more than 15 consecutive days.

Patients underwent surgical treatment of the obstruction either through insertion of a pigtail catheter under general anesthesia or by percutaneous drainage under ultrasound guidance. Intervention was done within the first 24 h from hospital admission. Six mL of blood was sampled after venipuncture of one forearm vein under aseptic conditions immediately before intervention and 12 h after intervention. Blood was collected into one sterile and pyrogen-free tube (Vacutainer, Becton Dickinson, Cockeysville Md) and centrifuged at room temperature. Serum was collected and stored at −80 °C before assayed for measurement of LPS, Ang-2 and interleukin (IL)-6. The primary endpoint of the study was to explore the factors associated with the change of LPS and Ang-2 after removal of the obstruction. The secondary endpoint was to explore how changes of LPS affect systemic inflammation as this is expressed by serum concentrations of IL-6.

2.2. Treatment of experimental sepsis with angiopoietin-2

Acute pyelonephritis was induced as already described by our group in 40 white male New Zealand rabbits of a mean weight of 3.5 kg [8,9]. Rabbits were kept in an isolated room with a controlled temperature of 24 °C and a day-night cycle of 8am–8pm. The study was approved by the Ethics Committee of ATTIKON University General Hospital and by the Veterinary Directorate of the Prefecture of Athens, Greece (license number K/2404/2009).

Briefly, after initial sedation with the intramuscular injection of 25 mg/kg of ketamine and 5 mg/kg of xylazine, through an upper midline abdominal incision, the peritoneal cavity was entered and the intestines were displaced to the left. The right ureter was recognized and ligated with a 3.0 suture just below the pelvis. A total of 1×10^7 cfu/kg of the *Escherichia coli* 15941 isolate at a volume of 0.1 mL, were injected by a 26-Gauge needle into the renal pelvis, proximal to the suture. The peritoneal cavity and the abdominal wall were closed in layers. Throughout the experimental procedure, anesthesia was maintained by the intramuscular administration of 15 mg/kg of xylazine at 30-min time intervals. Animals were then divided into two study groups: (i) controls ($n = 9$), administered bolus intravenously by a catheter inserted into the left ear vein one mL of water for injection (WFI) 90 min after bacterial challenge and (ii) Ang-2 ($n = 9$) administered bolus intravenously by the left ear vein catheter one hour after bacterial challenge 10 μg/kg of recombinant Ang-2 (R&D Inc., Minneapolis, USA) diluted into one mL WFI. The dose of Ang-2 was selected based on a previous study of our group [5]. Rabbits were resuscitated with the intravenous administration of 15 ml/h of N/S 0.9% through a catheter inserted into the left ear vein. After recovery, rabbits were transferred to their cages and given standard analgesia with paracetamol suppositories twice daily to reduce suffering. In these animals, one ml of blood was sampled by the right ear vein under aseptic conditions two and 24 h after bacterial challenge and transferred into one sterile and pyrogen-free tube (Vacutainer) for centrifugation and subsequent storage of serum at −80 °C. Survival was recorded for 21 days.

In separate experiments, the above model of acute obstructive uropathy and infection was repeated. Instead of *E. coli* a similar inoculum of the lower virulence isolate of *P. aeruginosa* 2 was injected at the right renal pelvis. Both of these isolates were used in previous experiments of our group allowing know their efficacy when used as bacterial challenge [8,9]. After assignment to treatment with WFI or Ang-2, 3.5 mL of blood was sampled by venipuncture under aseptic conditions from the right ear vein at 2 and 24 h after bacterial challenge; three mL was added into heparinized sterile tubes to be used for the isolation of peripheral blood mononuclear cells (PBMCs) and the remaining was collected into sterile and pyrogen-free tubes for centrifugation and subsequent storage of serum at −80 °C. At 48 h, rabbits were sacrificed with the intramuscular injection of 25 mg/kg of ketamine. After a midline abdominal incision under sterile conditions, the intestines were displaced to the left. Segments of 0.5 g of liver, spleen, right kidney and lung were cut by separate sterile blades and collected into separate sterile containers for tissue culture.

2.3. Laboratory procedures

PBMCs were isolated after gradient centrifugation of heparinized whole blood over Ficoll-Hypaque (Biochrom AG, Berlin, Germany). After three consecutive washings in ice-cold phosphate buffered saline pH 7.2 (Biochrom), PBMCs were counted in a Neubauer chamber after trypan blue exclusion of dead cells. PBMCs were distributed into wells of one 96-well plate of a final volume of 0.2 mL per well and incubated with RMPI 1640 enriched with 10% Fetal Bovine Serum (Biochrom), 2 mM glutamine and 10 mM pyruvate at a density of 2×10^6 PBMCs/mL for 24 h at 37 °C and 5% CO_2. Stimulation was performed without/with 10 ng/mL of the TLR4 agonist LPS of *E. coli* O55:B5 (Sigma Co, St. Louis, USA) and without/with 5 μg/mL of the TLR2 agonist Pam3Cys-SKKK (EMC Microcollections, Tübingen, Germany). At the end of the incubation, plates were centrifuged and the supernatants were collected and stored at −70 °C.

For the measurement of LPS, serum was diluted 1:10 with pyrogen-free water (BioWhitaler, Maryland, USA) and incubated for five minutes at 70 °C. LPS was then measured by the kinetic QCL-1000 LAL assay (BioWhitaker, lower detection limit 0.05 EU/mL).

Tissue segments were weighted and homogenized; one aliquot of 0.1 mL was diluted 1:10 into sterile sodium chloride four consecutive times. Another aliquot of 0.1 mL of each dilution was plated onto McConkey agar and incubated at 35 °C for a total period of three days. Plates were incubated at 35 °C and the number of viable colonies was estimated after multiplying with the appropriate factor of dilution. Identification of colonies was performed by the API20E system (BioMérieux, Paris, France). The number of viable cells was expressed as its $\log_{10}$ value in colony forming units (cfu)/g. The lower detection limit was 10 cfu/g.

Concentrations of IL-6 and Ang-2 were measured in serum by an enzyme immunosorbent assay (R&D Inc). The lowest limit of detection was 20 pg/mL for IL-6 and 5 pg/mL for Ang-2. TNFα was measured in supernatants of PBMCs in quadruplicate by a